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The Stress-inducted Proteins RTP801 and RTP801L Are Negative Regulators of the Mammalian Target of Rapamycin Pathway*

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The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays an essential role in cell growth control. mTOR stimulates cell growth by phosphorylating p70 ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E-binding protein 1 (4EBP1). The mTOR pathway is regulated by a wide variety of cellular signals, including mitogenic growth factors, nutrients, cellular energy levels, and stress conditions. Recent studies have proposed several mechanisms to explain how mTOR is regulated by growth factors and cellular energy levels. However, little is known as to how mTOR is regulated by stress conditions. We observed that two stress-induced proteins, RTP801/Redd1 and RTP801L/Redd2, potently inhibit signaling through mTOR. Our data support that RTP801 and RTP801L work downstream of AKT and upstream of TSC2 to inhibit mTOR functions. These results add a new dimension to mTOR pathway regulation and provide a possible molecular mechanism of how cellular stress conditions may regulate mTOR function.

A fundamental question in cell biology is how various extracellular cues can cause changes in translational output and hence the growth of the cell. The mammalian target of rapamycin (mTOR)[1] is a key regulator of translation that acts to stimulate protein synthesis by phosphorylating the ribosomal translation regulators p70 ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E-binding protein 1 (4EBP1) (reviewed in Refs. 1–4). mTOR is known to receive inputs from multiple signaling pathways and responds by increasing or decreasing protein synthesis appropriately. A prominent example of this phenomenon is how mTOR is stimulated by growth factors and the availability of nutrients, while it is inhibited by conditions such as low ATP levels, the absence of nutrients, or cellular stressors such as DNA damage or hypoxia. Regulation of protein synthesis by mTOR is responsible for controlling cell size and proliferation, and it has recently been shown that mTOR may also regulate other cellular processes such as cytoskeletal organization, mRNA turnover, transcription, and autophagy (1). Dysregulation of the mTOR pathway *in vivo* is associated with several inherited human syndromes, including tuberous sclerosis complex (TSC), the PTEN hamartoma tumor syndromes, and Peutz-Jeghers Syndrome (5, 6).

The tuberous sclerosis complex gene products, TSC1 and TSC2, are negative regulators of the mTOR signaling network (7). TSC1 and TSC2 form a complex in the cell, and the integrity of the TSC1/2 complex is essential for either protein to function. Cells or tissues lacking TSC1 or TSC2 display high levels of mTOR activation as measured by the activation status of S6K and 4EBP1, indicating that the TSC1/2 complex negatively regulates mTOR function (8–11). This inhibition occurs via the GAP (GTPase-activating protein) domain of TSC2. The target of the GAP activity of TSC2 is the small G protein Rheb, a potent positive upstream regulator of mTOR (12–14). Several studies have found that TSC2 stimulates GTP hydrolysis and inactivation of Rheb both *in vivo* and *in vitro* (15, 16).

Many of the signals known to impinge upon mTOR act through TSC2. For example, we have recently found that the AMP-activated protein kinase (AMPK) directly phosphorylates and activates TSC2, thereby inhibiting translation through mTOR (17). AMPK is a serine/threonine kinase that is activated in response to low energy conditions. Under such conditions of ATP depletion, AMPK phosphorylates numerous substrates to enhance catabolism and suppress anabolism (18). AMPK-mediated activation of TSC2 suppresses mTOR, as assayed by the inhibition of the phosphorylation of the mTOR targets S6K and 4EBP1, reduction in cellular protein synthesis, and a decrease in the overall size of cells (17). Therefore, the TSC2-mTOR pathway plays a critical role in the coordination between cellular energy levels and cell growth. In addition to low energy levels, it has been proposed that activation of AMPK is also responsible for coupling other stressors, such as osmotic stress, heat shock, or hypoxia to signaling through mTOR (19).

AKT is a pro-survival kinase that is activated by insulin stimulation and positively regulates mTOR signaling by phosphorylating and inactivating TSC2 (9, 20, 21). AKT may also stimulate mTOR independently of TSC2 phosphorylation (22). Recently, Reiling and Hafen (23) performed a genetic screen in *Drosophila* for genes that, when overexpressed, suppressed an mTOR hyperactivation phenotype downstream of AKT. Two hypoxia-induced proteins, termed *Scylla* and *Charybdis*, were identified from the genetic screen as negative regulators of the *Drosophila* TOR pathway. In this paper we investigate the mammalian orthologs of *Scylla* and *Charybdis*, RTP801 (also known as Redd1 and dig2) and RTP801L (also known as Redd2). RTP801 and RTP801L share ~50% sequence identity to each other but show little homology to other known proteins. We show that both RTP801 and RTP801L function as potent negative regulators of the mammalian target of rapamycin. Furthermore, we show that these proteins function upstream of

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[1] The abbreviations used are: mTOR, mammalian target of rapamycin; S6K, S6 kinase; 4EBP1, eukaryote initiation factor 4E-binding protein 1; TSC, tuberous sclerosis complex; GAP, GTPase-activating protein; AMPK, AMP-activated protein kinase; HA, hemagglutinin; RNAi, RNA interference; siRNA, short interfering RNA.

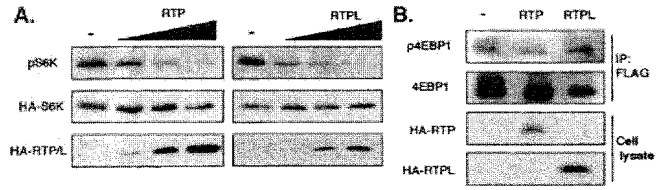

FIG. 1. **The stress-induced proteins RTP801 and RTP801L inhibit the mTOR targets S6K and 4EBP1.** *A*, RTP801 and RTP801L decrease the activation status of S6K in a dose-dependent manner. HA-S6K and HA-RTP801 (*HA-RTP*) or HA-RTP801L (*HA-RTPL*) were co-transfected into HEK293 cells, and the lysates were analyzed by Western analysis with a phospho-specific antibody against the mTOR phosphorylation site on S6K, T389. The levels of HA-S6K, HA-RTP801, and HA-RTP801L in the cell lysates was determined by immunoblotting with an anti-HA antibody. *B*, RTP801 and RTP801L decrease the activation status of 4EBP1. FLAG-4EBP1 and HA-RTP801 or HA-RTP801L were co-expressed in HEK293 cells, and anti-FLAG antibody was used to immunoprecipitate (*IP*) 4EBP1. The activation state of 4EBP1 was monitored with a dual phospho-specific antibody against the 4EBP1 activation sites T37/46. Levels of HA-RTP801 (*HA-RTP*) and HA-RTP801L (*IIA-RTPL*) were monitored via an HA immunoblot of the total cell lysate.

TSC2 and Rheb. Since RTP801 and RTP801L are up-regulated at the transcriptional level in response to a variety of stresses, including DNA damage, hypoxia, and glucocorticoid treatment (23–27), we speculate RTP801 and RTP801L may play critical roles in coupling certain extra- and intracellular cues to the regulation of translation through mTOR.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Materials—Anti- phospho-GSK3β (S9) was obtained from Upstate (Charlottseville, VA). Anti-TSC2 antibodies were from Santa Cruz Biotechnololgy (Santa Cruz, CA). Anti-FLAG was from Sigma, and anti-HA and anti-Myc were obtained from Covance (Philadelphia, PA). Anti-*Drosophila* S6K was the generous gift of M. Stewart (28), and anti-RTP801 antiserum was the generous gift of L. Ellisen (24). Anti-horseradish peroxidase-conjugated IgG secondary antibodies were obtained from Amersham Biosciences (Buckinghamshire, UK). All other antibodies were obtained from Cell Signaling.

HA-tagged S6K (αII), FLAG-tagged 4EBP1, and GST-Akt were described previously (9). Myc-tagged Rheb and Rheb-L64 were also described previously (29). RTP801 and RTP801L constructs were generated by PCR subcloning from IMAGE consortium clones 4302878 and 3877854 into the pCDNA3–3xHA and pPGS-cite-neo-HA vectors, between BamHI and EcoRI cloning sites. All newly made constructs were verified by DNA sequencing.

The AMPK inhibitor, commonly known as compound C, was obtained from Merck and was described previously (30). Intact cells were treated with a 40 μM concentration of the compound suspended in Me_2SO for 3 h preceding lysis. Rapamycin was purchased from Cell Signaling and was suspended in methanol. Negative controls for all experiments with pharmacological reagents included vehicle.

Cell Culture, Transfection, and Immunoprecipitation—Mammalian cell types were cultured and transfected as described previously (30). $TSC2^{+/-}$ and $TSC2^{-/-}$ MEFs were obtained from D. Kwiatkowski. Cells were cultured for ~48 h post-transfection unless otherwise noted, harvested in a mild lysis buffer, and subjected to SDS-PAGE and Western analysis as described previously (31). All immunoblots represent the best example of at least two repeated experimental trials. Immunoprecipitations were performed as described previously (31).

Retroviral Infection—Retroviral infection was performed by transfecting 293 Phoenix retrovirus packaging cells with empty vector or pPGS-CITE-neo-HA-RTP801 and RTP801L as described previously (17).

RNA Interference (RNAi)—RTP801, RTP801L, and TSC2 short interfering RNAi oligonucleotides were purchased from Dharmacon (Denver, CO). Each siRNA is a pool of four siRNAs directed against RTP801 or RTP801L. Oligonucleotides were transfected into HEK293 cells, and lysates were made ~48 h post-transfection. Double-stranded RNAi against *Scylla* and *Charybdis* was made and used to treat S2 cells according to the method of Worby *et al.* (32).

RESULTS AND DISCUSSION

To determine whether RTP801 and RTP801L function to suppress signaling through the mammalian target of rapamycin, we determined the effect of overexpressed RTP801 and RTP801L on the key mTOR targets S6K and 4EBP1 in HEK293 cells. Our data show that both overexpressed RTP801 and RTP801L cause a dose-dependent inhibition of phosphorylation of co-transfected S6K (Fig. 1*A*). The data also imply that RTP801L may be a more potent inhibitor of S6K than RTP801, since lower expression levels of RTP801L are required to inhibit S6K than RTP801 (Fig. 1*A* and data not shown). The RTP801- and RTP801L-mediated inhibition of co-expressed S6K was also observed in CHO cells (data not shown), indicating the effect is not specific to HEK293 cells. Similarly, RTP801 and RTP801L also inhibit the phosphorylation of co-expressed 4EBP1 (Fig. 1*B*). These results suggest that overproduction of RTP801 and RTP801L potently inhibit signaling through mTOR.

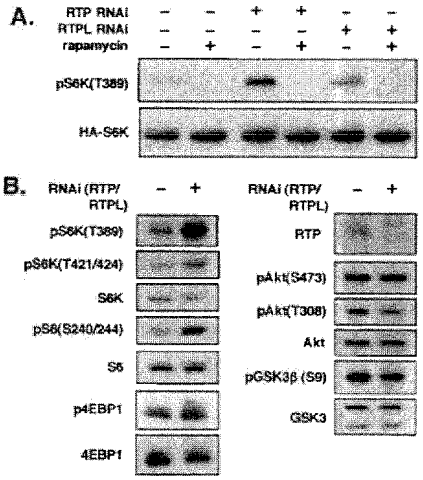

FIG. 2. **RTP801 and RTP801L RNAi increases S6K and 4EBP1 phosphorylation but has no effect on Akt.** *A*, down-regulation of RTP801 or RTP801L increases the activation status of S6K. Pools of siRNA oligonucleotides directed against RTP801 or RTP801L were co-transfected with HA-S6K into HEK293 cells. The level of increase in S6K activation was assayed via immunoblot with an antibody against the T389 activation site, and the level of HA-S6K was monitored with an anti-HA immunoblot. As a control, parallel samples were treated with 10 nM rapamycin for 30 min before lysis. *B*, down-regulation of RTP801 and RTP801L increases the activation of targets downstream of mTOR but has no effect on the activation status of the upstream protein Akt. RTP801 and RTP801L siRNA oligonucleotides were co-transfected into HEK293 cells and the activation status of S6K, S6 (the downstream target of S6K), 4EBP1, Akt, and GSK3β (a known downstream target of Akt) was determined with phospho-specific antibodies against residues known to be associated with the activation sites of each protein. Knockdown of the endogenous RTP801 protein was confirmed by immunoblotting with a polyclonal antibody against RTP801 (*RTP*).

Further supporting the physiological relevance of RTP801 and RTP801L in the regulation of S6K, we examined the effect of down-regulating RTP801 and RTP801L in HEK293 cells. Treatment of HEK293 cells with short interfering RNA oligouncleotides against RTP801 and RTP801L led to an increase in S6K phosphorylation on T389, and rapamycin treatment completely inhibited the effect of RTP801 or RTP801L RNAi (Fig. 2*A*). This increase was detected on both endogenous S6K (Fig. 2*B*) and on co-expressed S6K (Fig. 2*A*). We also observed an expected increase in phosphorylation of S6, the downstream target of S6K (Fig. 2*B*). Similar RNA interference experiments with *Scylla* and *Charybdis* were performed in *Drosophila* S2 cells. Down-regulation of either *Scylla* or *Charybdis* enhanced phosphorylation of the *Drosophila* S6K.[2] Taken together with the overexpression experiments, these results further support the role of RTP801 and RTP801L as potent negative regulators of signaling upstream of mTOR.

[2] M. N. Corradetti, K. Inoki, and K.-L. Guan, unpublished observations.

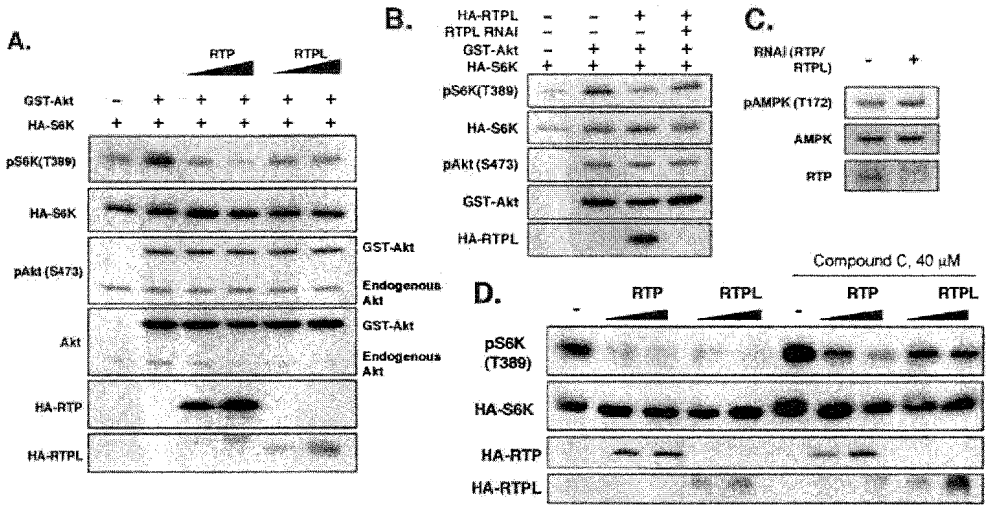

FIG. 3. **RTP801 and RTP801L function downstream of Akt and downstream or parallel to AMPK.** *A* and *B*, RTP801 and RTP801L inhibit Akt-mediated S6K activation and have no effect on the activation status of Akt. RTP801 (*RTP*) or RTP801L (*RTPL*) were co-transfected along with GST-Akt and HA-S6K into HEK293 cells, as indicated. Immunoblots were performed using the indicated antibodies. *B*, RTP801L RNAi inhibits the expression of HA-RTP801L. Since no antibody against RTP801L is available, evidence of down-regulation of the RTP801L protein was established by showing that siRNA oligonucleotides against RTP801L abolish expression of co-transfected HA-RTP801L but not the other indicated constructs. *C*, knock-down of RTP801 and RTP801L has no effect on the activation status of AMPK. siRNA oligonucleotides against HA-RTP801 and RTP801L were co-transfected into HEK293 cells, and the activation status of AMPK was determined via immunoblot for the AMPK activation site, T172. RTP801 (*RTP*) and AMPK expression was also monitored via immunoblot. *D*, RTP801 and RTP801L partially inhibit S6K in the presence of Compound C, an AMPK inhibitor. RTP801 (*HA-RTP*) or RTP801L (*HA-RTPL*) were co-transfected with HA-S6K into HEK293 cells and either treated with Compound C or vehicle, as indicated. Immunoblots were performed using the indicated antibodies.

To address how RTP801 and RTP801L inhibit signaling through mTOR, we sought to determine how RTP801 and RTP801L function in the context of known constituents of the mTOR pathway. The serine/threonine AKT is a key positive regulator of mTOR that functions by phosphorylating and inactivating the tuberous sclerosis gene product TSC2, a negative regulator of mTOR (9, 20, 21). Our results indicate that overexpression of RTP801 and RTP801L in HEK293 cells has little effect on the phosphorylation and activation state of a co-expressed Akt construct, although the basal activity of the AKT construct is sufficient to activate S6K (Fig. 3*A*). Consistently, RNAi oligonucleotides directed against RTP801 and RTP801L have little effect on the phosphorylation of endogenous Akt or a downstream target of Akt, GSK3β (Fig. 2*B*). Moreover, RTP801 and RTP801L inhibited AKT-induced S6K phosphorylation (Fig. 3*A*). The inhibitory effect of RTP801L expression on S6K phosphorylation was effectively reversed by co-transfection of RNAi oligonucleotides, and RTP801L siRNA also abolished the expression of co-transfected HA-RTP801L (Fig. 3*B*). Collectively, these results indicate that RTP801 and RTP801L likely act downstream of AKT in mammalian cells.

We also tested the relationship of RTP801 and RTP801L to AMPK, another known constituent of the mTOR pathway (17). To perform this experiment, we used a pharmacological inhibitor of AMPK (commonly known as Compound C) that specifically inhibits AMPK and not its upstream kinase LKB1 (30, 31). Overexpressed RTP801 and RTP801L were still able to inhibit S6K phosphorylation in the presence of Compound C, albeit not as fully as in the absence of the compound (Fig. 3*D*). In addition, siRNA directed against RTP801 and RTP801L did not have a significant effect on AMPK phosphorylation (Fig. 3*C*). Taken together, these data imply that RTP801 and RTP801L do not act upstream of AMPK. However, from these data we cannot distinguish whether RTP801 and RTP801L act downstream of AMPK or parallel to AMPK.

To determine the relationship between RTP801, RTP801L, and TSC2, we down-regulated the endogenous TSC2 protein with siRNA oligonucleotides. The results indicate that RTP801 and RTP801L were unable to fully inhibit S6K in the presence of TSC2 RNAi oligonucleotides (Fig. 4*A*). To further test the functional relationship between RTP801, RTP801L, and TSC2, we co-expressed RTP801 and RTP801L via retroviral infection in $TSC2^{-/+}$ and $TSC2^{-/-}$ cells. Our results show that co-expression of RTP801 and RTP801L cause a reduction in endogenous S6K phosphorylation in $TSC2^{+/+}$ cells but not $TSC2^{-/-}$ cells (Fig. 4*B*). Consistent with these data, RTP801 and RTP801L are unable to suppress S6K in the presence of overexpressed Rheb, the downstream target of TSC2 (Fig. 4*C*). The inability of RTP801 and RTP801L to suppress S6K was observed in the presence of either wild-type Rheb or Rheb L64, a more active mutant of Rheb. Therefore, RTP801 and RTP801L function upstream of TSC2 and Rheb to inhibit mTOR signaling.

While RTP801 and RTP801L function similarly to inhibit signaling through the mammalian target of rapamycin, it is not likely that they are regulated by all the same agonists, since the organization of their respective promoters are different. Significantly more is known about regulation of RTP801 than RTP801L, possibly due to the decreased stability of RTP801L relative to RTP801 (24). RTP801 transcription has been shown to be rapidly up-regulated by hypoxia via the hypoxia inducible factor HIF1 (26). It has also been shown that RTP801 is up-regulated by treatment with the glucocorticoid drug dexamethasone (27). Interestingly, glucocorticoids such as dexamethasone have been shown to cause a robust inhibition of S6K in cells that express the glucocorticoid receptor (33, 34), implying that the immunosuppresive effects of the mTOR-targeting drug rapamycin and glucocorticoid receptor-targeting drug dexamethasone may at least partially share a mechanism of action. Thus we speculate that since RTP801 (also called dexamethasone-induced gene 2, dig2) has been shown to be rapidly and stably up-regulated by dexamethasone (27), it is possible that dexamethasone-mediated inhibition of S6K works through a mechanism that requires RTP801.

In addition, it has been well established that in response to DNA damage, cellular transcription of many genes is dramatically reduced through a mechanism that requires the transcription factor p53. Much less work has been performed on the regulation of translation in response to DNA damage, but it has

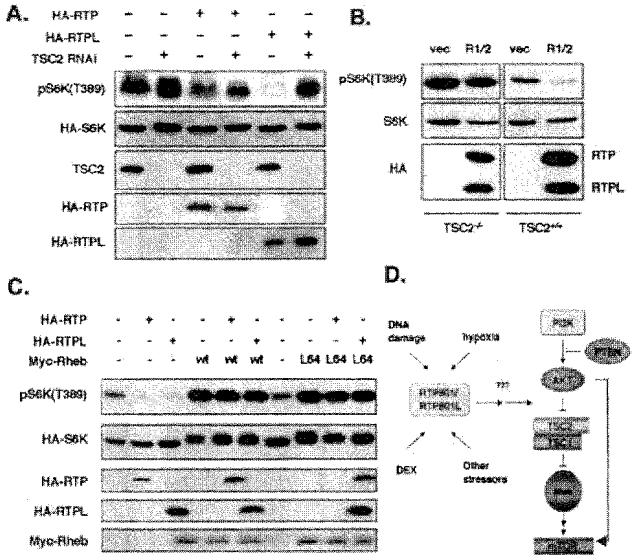

FIG. 4. **RTP801 and RTP801L function upstream of TSC2.** *A*, TSC2 is required for RTP801 or RTP801L to fully inhibit S6K. TSC2 was down-regulated in HEK293 cells by RNA interference in the presence or absence of co-transfected HA-RTP801 (*HA-RTP*) and RTP801L (*HA-RTPL*). The activation status of HA-S6K was monitored via immunoblot on T389, and the levels of TSC2, HA-S6K, and HA-RTP801 and RTP801L were determined as indicated. *B*, RTP801 and RTP801L are unable to inhibit S6K in cells null for TSC2. Wild-type or TSC2-null MEFs were infected with retroviruses containing either empty vector or HA-RTP801 (*HA-RTP*) and HA-RTP801L (*HA-RTPL*). Immunoblots were performed using the indicated antibodies. *C*, RTP801 and RTP801L are unable to inhibit S6K in the presence of overexpressed Rheb. HA-RTP801 or HA-RTP801L were co-transfected with HA-S6K into HEK293 cells in the presence or absence of Myc-Rheb or a more active mutant of Rheb, Myc-Rheb-L64. Immunoblots were performed using the indicated antibodies. *D*, a proposed model for the role of RTP801 and RTP801L in the mTOR pathway. RTP801 and RTP801L are proteins induced at the transcriptional level in response to a variety of stresses, including DNA damage, hypoxia, and dexamethasone (*DEX*). Through an unknown mechanism, RTP801 and RTP801L activate the TSC1/2 complex downstream of Akt. TSC2 GAP activity leads to a reduction in the GTP-bound, active form of the small GTPase Rheb and subsequently decreased signaling through the mTOR effectors S6K and 4EBP1.

been shown that several DNA damaging agents cause inhibition of signaling through mTOR (35, 36). Since it has also been shown that a variety of DNA damaging agents, including etoposide, ionizing radiation, and the DNA alkylating agent methyl methanesulfonate cause a rapid and stable increase in the transcription of RTP801 (24, 27), future studies should be aimed at determining whether RTP801 and/or RTP801L play any role in coupling the ability of the cell to sense DNA damage and the subsequent reduction in translation through mTOR.

It will be critical in the future to determine how the structure of RTP801 and RTP801L leads to inhibition of signaling through mTOR. RTP801 and RTP801L possess little detectable homology to known protein domains. It remains unclear whether these proteins possess catalytic function, whether they serve as adaptor proteins, or whether they have some other, unknown mechanism of action. It also remains to be seen whether RTP801 and RTP801L somehow affect TSC2 GAP activity. The fact that RNAi against either RTP801 or RTP801L elevated S6K phosphorylation more dramatically in cells cultured in older medium than fresh medium (data not shown) suggests that these two gene products may play a more prominent role in mTOR regulation under poor growth conditions as opposed to rich conditions.

Both the overexpression and down-regulation experiments described in this report demonstrate that RTP801 and RTP801L play a role to inhibit the mTOR pathway (Fig. 4*D*). It should be noted that, during the course of this study, similar observations were also made by Brugarolas *et al.* (37). Both studies indicate that RTP801 and RTP801L act upstream of TSC2, Rheb, and mTOR and possibly downstream of AKT. These results are consistent with genetic studies by Reiling and Hafen (23) who demonstrated that *Scylla* and *Charybdis*, the *Drosophila* homologs of RTP801 and RTP801L, down-regulate the TOR pathway in *Drosophila*. Thus, RTP801 and RTP801L should be considered as potential upstream mediators of signals caused by stressors to mTOR, but it is clear that the molecular mechanism of RTP801 and RTP801L in mTOR inhibition requires further investigation.

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